PCT

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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	U.S. APPLICATION NO. (If known, see 37CFR1.5) 10/019434

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/01887	July 3, 2000	July 1, 1999

TITLE OF INVENTION

USE OF THE *KRIT1* GENE IN THE FIELD OF ANGIOGENESIS

APPLICANTS FOR DO/EO/US

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Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau.
   - b. ☒ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   - a. ☒ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C. 154 (d)(4).
20. ☒ Other items or information:
    - a. ☒ Copy of cover page of International Publication No. WO 01/02604
    - b. ☒ Verification of a Translation (1 Sheet)
    - c. ☒ Sequence Listing (9 Sheets)
    - d. ☒ Statement Under 37 C.F.R. 1.821

U.S. APPLICATION NO. (If known, see 37CFR 1.5) 10/019434	INTERNATIONAL APPLICATION NO. PCT/FR00/01887	ATTORNEY'S DOCKET NUMBER 03715.0102

21. ☒ The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTOand all claims satisfied provisions of PCT Article 33 (1)-(4) **$100.00**

				ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).					$	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	24	- 20 =	4	x $18.00	$ 72.00	
Independent Claims	5	-3 =	2	x $84.00	$ 168.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+$280.00	$	
				TOTAL OF THE ABOVE CALCULATIONS =	$	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.					$	
				SUBTOTAL =	$1,130.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest priority date (37 CFR 1.492(f)).					$	
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Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					$	
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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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DATED: December 31, 2001

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USE OF THE *KRIT1* GENE IN THE FIELD OF ANGIOGENESIS

The present invention relates to a method for diagnosing cavernous angiomas, or cavernomas, for the detection of mutations in the *Krit1* gene. In particular, this detection is carried out using nucleotide sequences which are also the subject of the present invention. The invention also relates to the use of all or part of the *Krit1* gene for therapeutic purposes in the field of pro- or anti-angiogenesis.

Cavernomas are vascular malformations most commonly located in the central nervous system, but also in the retina, the liver, the kidneys, etc., and are characterized by capillary cavities which are abnormally enlarged without involvement of cerebral parenchyma (Russell et al.). The clinical symptoms comprise headaches, hemorrhages, epileptic fits and focal neurological deficiencies. The prevalence of cavernous angiomas is close to 0.5% in the general population (Otten et al.). These angiomas may be transmitted in a hereditary manner, in an autosomal dominant form, in close to 50% of cases (Rigamonti et al.). 3 locations (or loci) for cerebral cavernous malformations (CCMs) have been identified on the long arm of chromosome 7, the short arm of chromosome 7 and the long arm of chromosome 3 (7q, 7p and 3q, respectively). A considerable founder effect has been observed in the Hispano-American population, in which all the families are linked to the *CCM1* locus located on 7q (Rigamonti et al.; Dubovsky et al.; Günel et al.; and Craig et al.).

The inventors have recently established the genetic and clinical characteristics of cavernomas, and also the hereditary characteristics in a series of 57 French families (Labauge et al.). Neuroimaging investigations

have confirmed the high frequency of multiple lesions in hereditary cavernomas. A highly significant correlation has also been demonstrated between the number of lesions and the age of the patient, which strongly suggests the dynamic nature of these vascular malformations, also termed hamartomas. The genetic linkage analysis carried out in 36 of these families has shown that 65% of them are linked to the *CCM1* locus, with no founder effect (Laberge et al.).

The size of the genetic interval containing the *CCM1* locus had, in 1995, been reduced to 4 centimorgans, the *CCM1* locus being flanked by D7S2410 and D7S689 (Johnson et al.). Using essentially an *in silico* approach, the inventors established a physical and transcriptional map of the *CCM1* interval. Among the 53 transcriptional units mapped within the essential region, one of them corresponded to *Krit1*, a gene the product of which interacts with Rap1A (also termed Krev1), a member of the family of Ras genes involved in cell proliferation, differentiation and morphogenesis (Bos et al.). Using the SSCP technique and sequencing in combination, the inventors identified, in 8 unrelated *CCM1* families, mutations which very probably lead to a truncated Krit1 protein. The cosegregation of these mutations with the affected phenotype strongly suggests that *Krit1* is the protein mutated in the families suffering from cavernomas linked to the *CCM1* locus, and suggests that the Rap1A signal transduction pathway is involved in vasculogenesis and/or angiogenesis.

Using a previously published YAC contig and public sequence databases (The Washington University Chromosome 7 Project), the inventors constructed BAC/PAC contigs covering 90% of the *CCM1* interval, estimated at 1 600 Kb (Figure 1). 20 families comprising 179 potentially informative meioses, for which it had previously been shown that they had a

probability, a posteriori, of being linked to the *CCM1* locus of greater than 90%, were used to finely map this locus with polymorphic markers identified using the BAC/PAC sequences (Figure 1). A recombination event observed in an affected individual (family 27 in Labauge et al.) allowed the inventors to slightly reduce this interval, which is now flanked by M2456 (centromeric limit) and D7S689 (telomeric limit). The screening of public databanks, such as Gene Map of the Human Genome, Unigene and dBEST, allowed the inventors to map, within this interval, 574 *Expressed Sequence Tags* (ESTS), which were then regrouped into 53 putative transcriptional units comprising 6 already known genes: CDK6, HUMI.D14, KRIT1, PEX-1. mMTERF and Yotiao.

Krit1 had been identified during a screening intended to identify the proteins which interact with Rap1A/Krev1, a member of the Ras gene family (Serebriiski et al.). It encodes a 529 amino acid protein which comprises 4 ankyrin domains and interacts with Rap1A/Krev1 by means of its carboxyterminal region. It had already been indicated that the *Krit1* messenger RNA was expressed at low levels in many tissues, including the brain. Although the exact function of *Krit1* is still unknown, the inventors considered that it was a good candidate gene for *CCM1*, this being for several reasons. Rap1A/Krev1A was identified on the basis of its homology with *Dras3*, a Ras homologue in drosophila, and also on the basis of its antimitogenic activity in fibroblasts transformed with K-ras (Pizon et al., 1988/Kitayama et al., 1989). Although the physiological relevance of this anti-mitogenic effect observed *in vitro* has not yet been established *in vivo*, this has led to this protein being considered a Ras antagonist. A role for the Ras signaling pathway in vasculogenesis and angiogenesis has been strongly suggested by the vascular abnormalities observed in the murine models which are

knock-outs for the proteins involved in this pathway, for example the raf or GAP120 proteins (Henkemeyer et al., 1995; Wojnowski et al., 1997). In addition to this putative role as an Ras antagonist, Rap1A/Krev1 has been implicated in cellular differentiation and morphogenesis (Asha et al., 1999; Quarck et al., 1996; Pizon et al., 1988).

In other words, insofar as the trunctated Krit1 protein gives rise to an abnormality of angiogenesis accompanied by endothelial cell proliferation, it is reasonable to deduce therefrom that the *Krit1* gene may have a role in controlling angiogenesis.

Thus, in the context of the present invention, the inventors have shown that mutations in the *Krit1* gene, capable of giving rise to a truncated Krit1 protein, are responsible for the appearance of vascular abnormalities. These vascular abnormalities may affect various areas, including the brain and the skin, and take various forms (cavernomas, capillary-venous angiomas). The type of the lesions observed (development of abnormal vascular diseases) combined with the nature of the mutations observed (mutations leading to truncation of the protein) strongly suggest that this protein exerts a control over angiogenesis, which it may be possible to use therapeutically in the field of anti-angiogeneis, in particular in the tumor field.

The alignment of the *Krit1* cDNA with the BAC AC000020, one of the BACs located in the interval, has allowed the inventors to determine the genomic structure of *Krit1*. This gene is encoded by 12 exons, which are all included in the BAC AC000020. The inventors have illustrated the intronic oligonucleotide primers intended to amplify the exons (Table No. 1) and also the junction sequences (Table No. 2). These primers

were particularly tricky to develop because *Krit1* is rich in A and T bases, and are very specific for *Krit1*. Thus, a subject of the present invention is a nucleotide sequence chosen from the group comprising SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27 and SEQ ID No. 28.

By means of these primers, the inventors have been able to amplify all the exons. A set of 20 unrelated CCM patients belonging to families in which the HOMOG analysis showed a probability, *a posteriori*, of being linked to the *CCM1* locus of greater than 90%, has made it possible to screen mutations using an analysis which combines an approach of the Single Strand Conformation Polymorphism (SSCP) type, under 4 distinct conditions, and a sequencing approach.

The amplified products of 8 of these patients showed abnormal conformational variants which were not observed in any of the 50 control individuals. Analysis of the sequence of these amplimers revealed heterozygous mutations in these 8 patients (Table 3 and Figure 3). These mutations cosegregated with the diseased phenotype in the 8 families of these patients.

A subject of the present invention is also the use of at least one nucleotide sequence as defined above for detecting, from a biological sample, the presence of a mutation in the *Krit1* gene, preferably a mutation linked to the occurrence of vascular abnormalities as defined above. Preferentially, the biological sample is blood.

More particularly, pedigree 6 exhibits deletion of an A at nucleotide 1342, in exon 10. This deletion leads to a change in the reading frame and thus to a premature stop codon. In pedigree 10, substitution of C with T at nucleotide 1283 in exon 10 leads to the replacement of a glutamine with a stop codon. Pedigree 58, itself, shows insertion of a C after nucleotide 1271, also in exon 10, which leads to a change in the reading frame and a premature stop codon. Pedigree 41 shows substitution of G with A at nucleotide 615, which leads to the replacement of a tryptophan with a premature stop codon in exon 5. Pedigree 42 exhibits a 4 bp deletion (nucleotides 681-684) in exon 6, which gives rise to a premature stop codon. Pedigree 35 exhibits a 26 bp deletion (nucleotides 1012-1037) in exon 8, this deletion causing a change in the reading frame and a premature stop codon at codon 332. Pedigree 18 exhibits insertion of a C in exon 2 after nucleotide 247, this insertion leading to a change in the reading frame and a premature stop codon. Pedigree 19 shows substitution of a G with an A at nucleotide 261, this substitution causing a change in the reading frame and also a premature stop codon at codon 79.

The SSCP analyses of the affected and unaffected members have shown perfect cosegretation of the mutations with the affected phenotype in each of these 8 pedigrees (Figure 3).

Thus, the nucleotide sequences in accordance with the present invention are used to detect a mutation in at least one exon of the *Krit1* gene. More particularly, these nucleotide sequences may be used in pairs, given their specificity for an exon, according to the following distribution:

- SEQ ID No. 1/SEQ ID No. 2 for exon 1,
- SEQ ID No. 3/SEQ ID No. 4 for exon 2,

- SEQ ID No. 5/SEQ ID No. 6 for exon 3,
- SEQ ID No. 7/SEQ ID No. 8 for exon 4,
- SEQ ID No. 9/SEQ ID No. 10 for exon 5,
- SEQ ID No. 11/SEQ ID No. 12 for exon 6,
- SEQ ID No. 13/SEQ ID No. 14 for exon 7,
- SEQ ID No. 15/SEQ ID No. 16 for exon 8,
- SEQ ID No. 17/SEQ ID No. 18 for exon 9,
- SEQ ID No. 19/SEQ ID No. 20 for exon 10,
- SEQ ID No. 21/SEQ ID No. 22 for exon 10,
- SEQ ID No. 23/SEQ ID No. 24 for exon 11,
- SEQ ID No. 25/SEQ ID No. 26 for exon 12,
- SEQ ID No. 27/SEQ ID No. 28 for exon 12.

Advantageously, the detection of a mutation in Krit1 is preceded by amplification of the exon in which the mutation is being sought, and this amplification may be carried out by PCR or PCR-like amplification.

The truncating nature of these mutations, their absence in the healthy controls and their consegregation with the affected phenotype strongly suggest that they are mutations which are deleterious in these families.

The inventors did not detect any abnormal SSCP conformational variants in 12 of the 20 families tested. Several hypotheses may be put forward to explain this. SSCP is not 100% sensitive, even when several types of condition are used, as was the case here. Interestingly, none of these abnormal conformational variants was observed in the first screening, which was carried out at 20°C without glycerol. In addition, it would not have been possible to detect, using this approach, the deletions which would take away the regions containing the sequences which hybridize with the primers. Finally, some of these families, although showing a high probability of being linked to the *CCM1* locus, may, in fact, be linked to one of the other *CCM* loci.

A subject of the present invention is also a method for genotypically diagnosing vascular abnormalities in an individual, comprising taking a biological sample from said individual, and also detecting the presence of a mutation in the *Krit1* gene by analyzing the sequence of nucleic acids present in said sample, such a mutation being linked to the occurrence of vascular abnormalities. The nucleic acid sequence analyzed may be, indifferently, genomic DNA, cDNA or mRNA. The analysis may be carried out by hybridization, by sequencing or by electrophoretic migration, in particular by SSCP or DGGE (denaturing gradient gel electrophoresis). The detection of these mutations may also be carried out using methodology which makes it possible to directly detect the presence of the truncated protein, for example the "Protein Truncation Test" methods (in vitro translation of cDNA reverse transcripts, followed by revelation of the protein with antibodies or after labeling the protein using a labeled amino acid). Finally, the search for mutations may be carried out by direct analysis of the cDNA reverse transcript prepared from total RNAs (in particular originating from cells transformed with the EBV virus, cells in which the authors have shown the expression of the Krit1 transcript).

Advantageously, all or part of the nucleic acid sequence corresponding to the *Krit1* gene is amplified prior to detecting the presence of a mutation, this amplification possibly being carried out by PCR or PCR-like amplification. Entirely preferentially, this amplification may be carried out using primers chosen from the nucleic acid sequences in accordance with the present invention, for example used according to the abovementioned distribution.

The main question is therefore to understand how these mutations were able to lead to cavernomas. Little is,

in reality, known about the nature of these lesions, which are considered to be vascular malformations or hamartomas. It would appear that the period when these malformations appear during embryonic life is not entirely clear. In addition, in certain cases, particularly in familial cases, progressive extension of these hamartomas has been described: it has been suggested that these lesions may express factors and/or receptors involved in angiogenesis (Rothbart et al., 1996; Notelet et al., 1997).

It should be pointed out that the inventors have observed, in four families (Labauge et al., 1999) that cutaneous malformations (also termed angiomas) may segregate with cerebral cavernomas.

All the mutations reported herein would, if they were translated, produce truncated Krit1 proteins which would be deleted of the region which interacts with Rap1A/Krev1.

The exact functions of Rap1A/Krev1 have not been entirely elucidated. This member of the Ras GTPase family is expressed ubiquitously, particularly in neutrophils, platelets and the brain; it is located in the endocytic/lysosomal compartments. Rap1A has been described as interacting with B-Raf, which is quite interesting given the massive endothelial apoptosis observed in mice deficient in B-Raf (Wojnowski et al., 1997). *In vivo*, studies on lower eukaryotes, such as yeasts and drosophila, have recently given some indications concerning the functions of Rap1A in differentiation and morphogenesis (Asha et al.).

The interaction of Krit1 and Rap1A suggests that Krit1 may either regulate Rap1A or be an effector of Rap1A (Bos et al.). The mutations reported herein may result either from a dominant negative effect or from a loss

of function. The observation of families exhibiting complete deletions of Krit1 would be a strong argument in favor of this hypothesis. Moreover, the fact that the sporadic forms of cavernomas manifest themselves mainly as a single lesion, and that the familial forms manifest themselves not multiple lesions, strongly suggests that these lesions follow the *"Knudson double hit"* rule (Knudson 1971) and that a complete loss of Krit1 function may be necessary for the appearance of cavernomas.

In other words, in the dominant forms of the disease, a first mutation, present in all the cells of the organism in the heterozygous state, would be present. The appearance of the cavernomatous lesions would be conditioned by the occurrence of a second mutation affecting the other allele of this gene, this mutation occurring somatically. In the sporadic forms most studied to date, the individual exhibits no germinal mutation and the single lesion is thought to result from two mutations which have occurred in the same cell.

However, sporadic forms of cavernomas other than those described above are thought to exist. The inventors have, in fact, demonstrated a sporadic form which manifests itself as multiple lesions and results from a *de novo* mutation in the *Krit1* gene, probably in a germinal cell of one of the two parents of the patient affected (data not shown).

In summary, the data reported herein strongly suggest that the truncating mutations of Krit1 are responsible for the appearance of the cerebral cavernomas observed in the *CCM1* families, but also in certain sporadic forms, underlining the putative role of the Rap1A signaling pathway in these mechanisms.

Among the therapeutic applications with which the present invention is concerned, there may be various types of vascular malformations, vascular dysplasias, angiomas and/or any situation in which abnormal angiogenesis exists.

Thus, a subject of the present invention is also the use of the *Krit1* gene, or of a sequence derived from this gene, for producing a medicinal product, or its use in an approach of the gene therapy type intended to control or inhibit angiogenesis, in particular by over-expression, *in situ*, of the *Krit1* gene or a sequence derived from this gene.

The expression "sequence derived from this gene" is intended to mean any normal or mutated sequence, or portion of sequence, of the *Krit1* gene, which exhibits an activity similar and comparable to the total functional reference sequence.

A subject of the present invention is also a vector for expression in a suitable host cell, comprising the sequence of the *Krit1* gene or a sequence derived from this gene (the derived sequence is defined above). When the intention would be to repress abnormal angiogenesis, it may be advantageous to overexpress the sequence in question and, for this reason, the vector in accordance with the invention advantageously comprises the elements required for this overexpression.

In particular, the vector in accordance with the invention may be intended for gene therapy and, when the intention would be to limit its site of action, this vector may comprise a sequence for the tissue-specific targeting and/or expression of the sequences which it comprises.

Finally, the subject of the present invention is a therapeutic composition comprising, as active principle, all or part of the normal or modified Krit1 protein, so as to substitute, for example, for a truncated protein and compensate for the deficiency. The active principle may also be a vector as described above.

Figure 1 represents the genetic, physical and transcriptional map of the *CCM1* locus.

Figure 1a represents the genetic map of the *CCM1* locus. This locus was previously defined by the D7S2410-D7S689 interval. The reduced MS2456-D7S689 genetic intervals are indicated by horizontal square brackets. The microsatellites already published are boxed. The new microsatellites are identified by bold characters. Some of the STSs are also shown. The STS sWSS 1703 corresponds to nucleotides 393-658 of *Krit1*. The markers between the vertical square brackets are less than 1 kb apart.

Figure 1b represents the physical and transcriptional map of the *CCM1* locus. BAC contigs are distributed over the *CCM1* interval. The BAC AC000120 is represented by the thickest line. The overlaps with either the STSs or the microsatellite markers are indicated by small vertical bars. The black arrow corresponds to *Krit1*, the white arrows correspond to well-characterized human genes and the empty arrows correspond to genes exhibiting strong homologies with genes from other species (not characterized in humans).

Figure 2 represents the cosegregation of the conformational variants with the disease phenotype, within 8 pedigrees (SSCP).

The empty symbols denote the individuals whose MRI examination of the brain is normal, the half-full black

symbols correspond to asymptomatic patients exhibiting cavernomas on the MRI examination, and the full black symbols correspond to symptomatic patients exhibiting cavernomas on the MRI; the sign "?" corresponds to the individuals having an unknown status, and the sign "\" corresponds to deceased patients. The deceased patients or patients with unknown status were not tested for the mutation but are represented in the interests of clear understanding of the familial structures. The abnormal bands are indicated with an arrow.

Figure 3 illustrates the *Krit1* mutations.

Figure 3a represents the structure of the *Krit1* gene and of the corresponding putative protein. "ns" signifies nonsense, "del" signifies deletion, and "ins" signifies insertion. For the insertions, the nucleotide number corresponds to the nucleotide preceding the insertion. The expression "Krev Interacting Region" corresponds to the region (amino acids 483-529) for which the deletion destroys the interaction with Krev during the double hybrid test in yeast.

Figure 3b represents the *Krit1* mutations identified in the 8 pedigrees mentioned above. The arrows indicate the mutation sites. WT signifies wild-type sequence, and MT signifies mutant sequence. In pedigree 42, the chromatogram and the sequence presented correspond to the negative strand; the positive strand showed complete superposition of the normal and abnormal sequences and did not allow good visualization of the start site of the deletion.

EXAMPLES

MATERIALS AND METHODS

Patients

20 unrelated patients belonging to families who were known to exhibit cavernomas in a hereditary manner freely consented to take part in the study (Labauge et al.).

Analysis of this panel of families with the HOMOG test showed that these families had a probability, *a posteriori*, of being linked to the *CCM1* locus of greater than 90% (Laberge et al.).

An approach combining molecular biology and bioinformatics was used to establish the physical and transcriptional map of the *CCM1* locus. After validation of a contig of YACs previously published by Johnson et al. (1995), the authors positioned in the interval, by PCR and using an *in silico* approach, 574 EST (Expressed Sequence Tags) which they grouped into 53 groups.

Reduction of the genetic interval

12 polymorphic microsatellite markers covering the D7S2410-D7S689 interval were selected for the linkage analyses. 7 of them were previously known: D7S2410, D7S2409, D7S1813, D7S1789, D7S646, D7S689 and D7S558, and were used by several teams (Günel et al. in *Neurosurgery*, Craig et al., Labauge et al., Laberge et al.). The last ones, MS65, MS2453A, MS2456A, MS119 and MS120, were identified by the inventors on the basis of the sequence database for the BACs mapped in the interval.

Detection of mutations and identification

On the basis of the comparison of the sequences of the Krit1 cDNA and of the BAC AC 00000120, the inventors determined 14 sets of primers in order to amplify the 12 exons and the exon/intron junction sites of *Krit1* using genomic DNA. PCR reactions were carried out as follows: after a first initial denaturation step at 94°C (4 min), 30 amplification cycles consisting of steps at 94°C (15 s), an optimized hybridization temperature of between 45°C and 55°C (15 s) and 72°C (15 s), followed by a final extension step at 72°C (10 min). The PCR products were subjected to electrophoresis under 4 types of condition (10% acrylamide with or without glycerol at 4°C and 20°C) on a Mighty Small II apparatus (Pharmacia-Biogen) used under conditions of constant current of 35 mA. Conformational variants were revealed with silver (Silver Stain Plus kit, Biorad). Amplimers showing an atypical SSCP band pattern were sequenced (AB1377, Perkin Elmer). All the mutations detected during the sequencing were tested for their cosegregation with the diseased phenotype, using an SSCP approach.

TABLE 1: PRIMERS

EXON	SENSE PRIMER	REVERSE PRIMER	AMPLIMER SIZE
1	GAGCGGATAAAAACTAAT (SEQ ID No. 1)	GAGCTAAAATTCATTCAA (SEQ ID No. 2)	205
2	GCTCTTAATGGGTTTTTG (SEQ ID No. 3)	AGCAATGTGGAGTAAAAC (SEQ ID No. 4)	183
3	TTTGGAATGAGAACAGTC (SEQ ID No. 5)	GTCCTGTTGTATTTTTCA (SEQ ID No. 6)	265
4	GTTGTTGTTTTTTGTTTG (SEQ ID No. 7)	ACCTGGAAAATAACTTAC (SEQ ID No. 8)	208
5	ATGTAATGCCTTTTTTCC (SEQ ID No. 9)	ATGCCTGGCTCTAACTAT (SEQ ID No. 10)	181
6	TTGTTAGATTGTGATGTA (SEQ ID No. 11)	AACATAATAAAAACTTTC (SEQ ID No. 12)	257
7	TTTATAAAAGGAATGATG (SEQ ID No. 13)	TCAACTCAAACCATATCA (SEQ ID No. 14)	335
8	TGTAGCCTAATAACCAAA (SEQ ID No. 15)	AGCATAGCACAAGACCAT (SEQ ID No. 16)	243
9	GGTGAAGTTTTTAATATG (SEQ ID No. 17)	CAATAGTTTATGAAGTCC (SEQ ID No. 18)	213
10	ATATTTACAAAGGCAAGC (SEQ ID No. 19) TGGTACATTTTCCTTTCA (SEQ ID No. 21)	TGACATGATTGGTAAAAA (SEQ ID No. 20) CTTTATGATTGCTGGGGC (SEQ ID No. 22)	180 201
11	GGTGAAGTTTTTAATATG (SEQ ID No. 23)	CAATAGTTTATGAAGTCC (SEQ ID No. 24)	205
12	AATAGATAGGGAACTGCC (SEQ ID No. 25) TAATGCCCACTGAAAGAA (SEQ ID No. 27)	GTGGCTTGAGTAACAGTT (SEQ ID No. 26) GGCTGGTCTTGAACTCTG (SEQ ID No. 28)	234 199

TABLE 2: SEQUENCES OF THE INTRON-EXON JUNCTIONS

EXON	POSITION ON THE CDNA	SIZE	SEQUENCE	POSITION ON BAC AC000120
1	8-133	126	atcaggtcag ACAGAAAACT...TACAAATCGG gtaagagttg (SEQ ID No. 29) (SEQ ID No. 30)	127165-127040
2	134-249	116	ccctttctag GTAGATAAAG...CAGAAGACAA gtactgtttc (SEQ ID No. 31) (SEQ ID No. 32)	126561-126445
3	250-393	144	taatgattag GGAACGACAG...ATGCATGCTG gtaaatggaa (SEQ ID NO. 33) (SEQ ID No. 34)	126228-126086
4	394-550	157	ttttatacag GTATGGAAAA...AACGGATAGA gtaagttatt (SEQ ID No. 35) (SEQ ID No. 36)	118319-118163
5	551-657	107	acatttctag CATATAACAG...TAACAAACCA gtaagaatta (SEQ ID No. 37) (SEQ ID No. 38)	117464-117357
6	658-815	157	tttcttgtag TATGAAAAAG...GAAAACCTCA gtaagaaagt (SEQ ID No. 39) (SEQ ID No. 40)	114615-114459
7	816-967	152	tgtttttcag GCCTTCAACT...TGAAAAACAG gtttgcttgg (SEQ ID No. 41) (SEQ ID No. 42)	113690-113539
8	968-1134	168	ttcctttaag ATTGAAGACC...GTTTCCTAAA gtaagtattt (SEQ ID No. 43) (SEQ ID No. 44)	106414-106248
9	1135-1222	88	gtgcttacag TGAAGAAAAT...TGAATACAAG gtaagctgtt (SEQ ID No. 45) (SEQ ID No. 46)	105616-105529
10	1223-1429	207	ttgtttttag AATCTCAGTA...GGAAACTAAG gtagattttc (SEQ ID No. 47) (SEQ ID No. 48)	105038-104832
11	1430-1546	117	tatgttgcag GCTTTACTCA...TACAAAACAG gtaagtatca (SEQ ID No. 49) (SEQ ID No. 50)	93060-92942
12	1547-2004*	458*	tactttgtag GCTCTGGTCG* (SEQ ID No. 51)	92441-91984*

* Exon 12 not entirely determined since contains *Alu* sequences

TABLE 3: MUTATIONS IN *KRIT1*

PEDIGREE	MUTATIONS IN THE GENOMIC DNA	AMINO ACID MUTATIONS	
6	Deletion (A) nt 1342 Exon 10	Amino acid changes after AA 438	Normal (WT):IF (438) TKASPSNHKVIPVIVG... Mutant (MT): IF (438) TIRTAPAIIKSSLM*stop Codon
10	Missense (C→T) nt 1283 Exon 10	Amino acid changes after AA 420	WT:FL (420) QN... MT:FL (420)* stop codon
18	Insertion (C) after nt 247 Exon 2	Amino acid changes after AA 74	WT:ED (74) KERQWDD... MT:ED (74) QGTTVGR * stop codon
19	Missense (G→A) nt 261 Exon 3	Amino acid changes after AA 79	WT:RQ (79) WVDD... MT:RQ (79) * stop codon
35	Deletion (26 bp) nt 1012 Exon 8	Amino acid changes after AA 328	WT:EA (328) RYNLLKGFYTAPDAKL... MT:EA (328) SSS * stop codon
41	Missense (G→A) nt 615 Exon 5	Amino acid changes after AA 197	WT:NN (197) WEEAA... MT:NN (197) * stop codon
42	Deletion (GAAT) nt 681-684 Exon 6	Amino acid changes after AA 217	WT:IY (217) RMDGSYRSVELK... MT:IY (217) RMGHIVLLN * stop codon
58	Insertion (C) after nt 1271 Exon 10	Amino acid changes after AA 415	WT:LQ (415) RMFLQNCQIFTKASPSNHKV... MT:LQ (415) HVLTQLTDIKGYKPQQS * stop codon

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CLAIMS

1. A nucleotide sequence chosen from the group comprising SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27 and SEQ ID No. 28.

2. A use of at least one nucleotide sequence as claimed in claim 1, for detecting, using a biological sample, the presence of a mutation in the *Krit1* gene.

3. The use of at least one nucleotide sequence as claimed in claim 2, for detecting, from a biological sample, the presence of a mutation in the *Krit1* gene, linked to the occurrence of cavernomas.

4. The use as claimed in either of claims 2 and 3, characterized in that the detection of the mutation takes place in at least one exon of the *Krit1* gene.

5. The use as claimed in claim 4, characterized in that a pair of sequences is specific for an exon according to the following distribution:
   - SEQ ID No. 1/SEQ ID No. 2 for exon 1,
   - SEQ ID No. 3/SEQ ID No. 4 for exon 2,
   - SEQ ID No. 5/SEQ ID No. 6 for exon 3,
   - SEQ ID No. 7/SEQ ID No. 8 for exon 4,
   - SEQ ID No. 9/SEQ ID No. 10 for exon 5,
   - SEQ ID No. 11/SEQ ID No. 12 for exon 6,

- SEQ ID No. 13/SEQ ID No. 14 for exon 7,
- SEQ ID No. 15/SEQ ID No. 16 for exon 8,
- SEQ ID No. 17/SEQ ID No. 18 for exon 9,
- SEQ ID No. 19/SEQ ID No. 20 for exon 10,
- SEQ ID No. 21/SEQ ID No. 22 for exon 10,
- SEQ ID No. 23/SEQ ID No. 24 for exon 11,
- SEQ ID No. 25/SEQ ID No. 26 for exon 12,
- SEQ ID No. 27/SEQ ID No. 28 for exon 12.

6. The use as claimed in one of claims 2 to 5, characterized in that the detection of a mutation is preceded by amplification of the exon in which the mutation is being sought.

7. The use as claimed in claim 6, characterized in that the amplification is carried out by PCR or PCR-like amplification.

8. A method for genotypically diagnosing cavernomas in an individual, characterized in that a biological sample is taken from said individual, and in that the presence of a mutation in the *Krit1* gene is detected by analyzing the nucleic acid sequence present in said sample, such a mutation being linked to the occurrence of cavernomas.

9. The diagnostic method as claimed in claim 8, characterized in that the nucleic acid sequence is genomic DNA, cDNA or mRNA.

10. The diagnostic method as claimed in either of claims 8 and 9, characterized in that said analysis is carried out by hybridization.

11. The diagnostic method as claimed in one of claims 8 to 10, characterized in that said analysis is carried out by sequencing.

12. The diagnostic method as claimed in either of claims 8 and 9, characterized in that said analysis is carried out by electrophoretic migration, and more particularly by SSCP or DGGE.

13. The diagnostic method as claimed in either of claims 8 and 9, characterized in that said analysis is carried out by methodology aimed at detecting the truncation of a protein.

14. The diagnostic method as claimed in one of claims 8 to 13, characterized in that all or part of the nucleic acid sequence corresponding to the *Krit1* gene is amplified prior to detecting the presence of a mutation.

15. The diagnostic method as claimed in claim 14, characterized in that the amplification is carried out by PCR or PCR-like amplification.

16. The diagnostic method as claimed in claim 15, characterized in that the primers for carrying out the amplification are from the sequences defined in claim 1, preferably in claim 5.

17. The use of the *Krit1* gene, or of a sequence derived from this gene, for preparing a medicinal product intended to control or inhibit angiogenesis.

18. The use as claimed in claim 17, characterized in that the medicinal product is intended for gene therapy.

19. A vector for expression in a suitable host cell, characterized in that it comprises a sequence of the *Krit1* gene or a sequence derived from this gene.

20. The expression vector as claimed in claim 19, characterized in that it comprises the elements required for the overexpression of the sequence.

21. The vector as claimed in claim 19 or 20, intended for use as claimed in either of claims 17 and 18.

22. The vector as claimed in one of claims 19 to 21, characterized in that it comprises a sequence for tissue-specific targeting and/or expression.

23. A therapeutic composition, characterized in that it comprises, as active principle, at least all or part of the normal or modified Krit1 protein.

24. The composition as claimed in claim 23, characterized in that the compound is a vector as claimed in one of claims 19 to 22.

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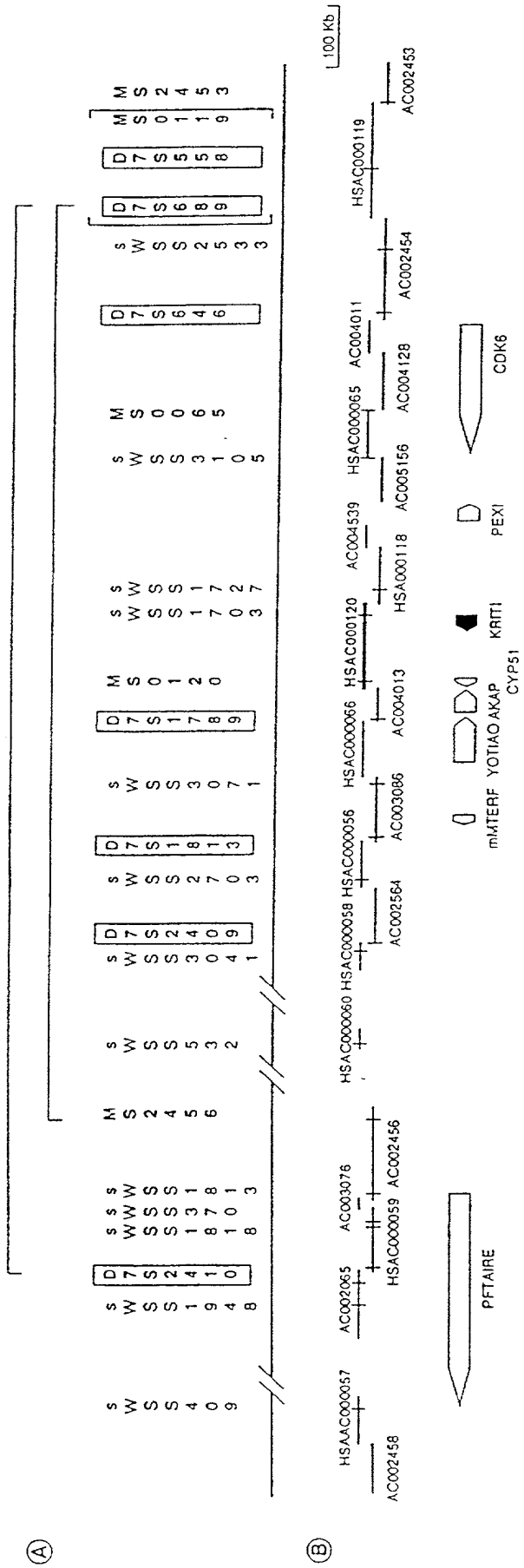
(54) Title: USE OF THE *KRIT1* GENE IN ANGIOGENESIS

(54) Titre: UTILISATION DU GENE *KRIT1* DANS LE DOMAINE DE L'ANGIOGENESE

(57) Abstract: The invention concerns a method for diagnosing cavernous angiomas for detecting mutations in the *Krit1* gene. More particularly, said detection is performed by using nucleotide sequences. The invention further concerns the use of the *Krit1* gene for therapeutic purposes in the field of angiogenesis.

(57) Abrégé: La présente invention a pour objet une méthode de diagnostic des cavernomes pour la détection de mutations dans le gène *Krit1*. En particulier, cette détection est mise en oeuvre au moyen de séquences nucléotidiques également objet de la présente invention. Elle concerne également l'utilisation du gène *Krit1* à des fins thérapeutiques dans le domaine de l'angiogenèse.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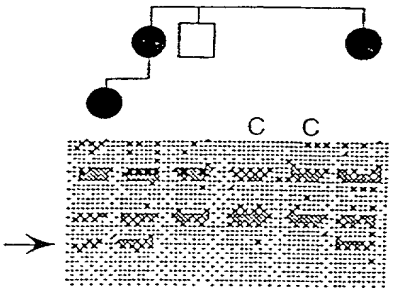
Ⓐ
Ⓑ

FIGURE 1

Pedigre 6



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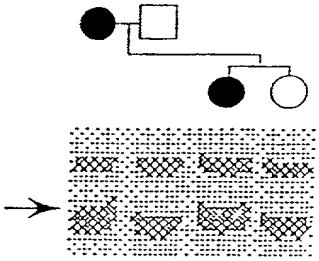

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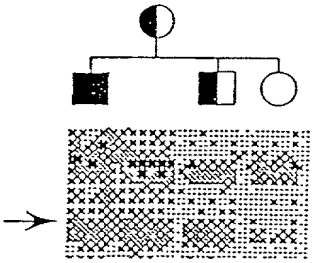
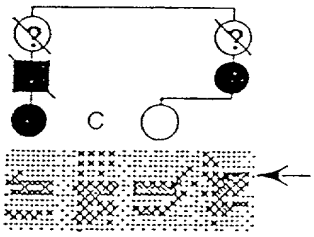
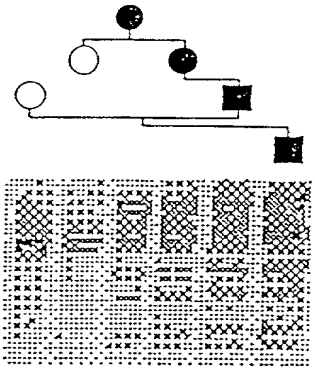


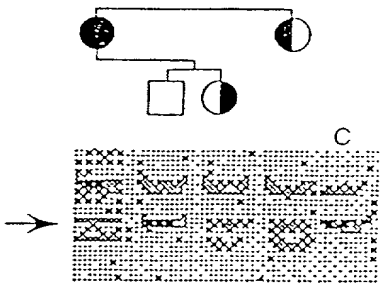
Pedigre 19



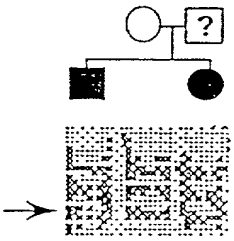
Pedigre 35



Pedigre 41



Pedigre 42



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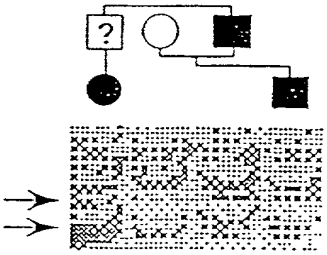
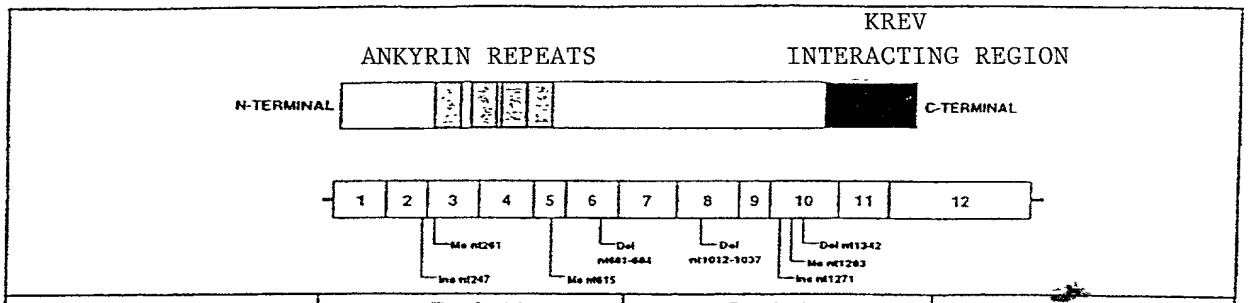


FIGURE 2



Ped. 6	Ped. 10	Ped. 18	Ped. 19
Exon 10 1342 Del (A) WT TKASPSNHKVIPVIVG MT TIRTAPAIIKSSLM*STOP	Exon 10 1283 C -> T WT Q MT *STOP	Exon 2 247 Ins (C) WT KERQWDD... MT QGTTVGR*STOP*	Exon 3 261G -> A WT W MT *STOP
WT TATTTACAAAGGCAAGC MT TATTTACAAGGCAAGC	WT CTTACAGAATT MT CTTATAGAATT	WT GAAGACAAGT[illegible]GT MT GAAGACCAAGTACTG	WT GTGGGTAGATGA MT GTGAGTAGATGA
Ped. 35	**Ped. 41**	**Ped. 42**	**Ped. 58**
Exon 8 1012 Del (26bp) WT RYNLLKGFYTAPDAKL... MT SSS*STOP	Exon 5 615 G -> A WT WEEA... MT *STOP	Exon 6 681Del (GAAT) WT RMDGSYRSVELK... MT RMGHIVILLN*STOP	Exon 10 1271 Ins (C) WT RMNFLQNCQIFTKASPSNH... MT HVLTQLTDIKGYKPQQS*STOP
WT AAGCCAGATATAATTT MT AAGCCAGCAGCTCCTG	WT AACTGGGAAGA MT AACTAGGAAGA	WT CATCCATTCTGTATA MT CATCCTGTATATTCG	WT TTCAGCGCATGTTCTTA MT TTCAGCCGCATGTTCTT

FIGURE 3

Attorney Docket No. 03715.0102

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: USE OF THE KRIT1 GENE IN THE FIELD OF ANGIOGENESIS the specification of which ☐ is attached and/or ☒ was filed on 03 JULY 2000 as ~~United States Application Serial No.~~ _____ or PCT International Application No. PCT/FR00/01887 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C.
FRANCE	99/08504	01 JULY 1999	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)
PCT/FR00/01887	03 JULY 2000	Pending

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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